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DEVELOPMENT AND APPLICATION OF NUCLEIC ACID HYBRIDIZATION TECHNIQUES TO ARBOVIRUS SURVEILLANCE AND DIAGNOSIS

ANNUAL REPORT

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Nucleic acid hybridization techniques have been developed to detect dengue and LaCrosse arbovirus RNA in cells and in cell and tissue suspensions. Probes are comprised of cDNAs of portions of the respective virus genomes cloned into plasmids. Nonisotopic probes are prepared by nick translating the plasmid using biotinylated nucleotides. The presence of specific hybrids can then be detected immunologically using anti-biotin antibodies followed by signal amplification with an enzyme immunoassay. In situ hybridization techniques have been developed to detect the site and temporal onset of S RNA synthesis in cell cultures using a biotinylated cDNA of the S RNA as a probe. S RNA was detectected in the perinuclear region of BHK 21 cells by 4 hours post-infection; by 24 hours hybrids were found throughout the cytoplasm. A similar probe has been used to detect LaCrosse virus RNA in pools of infected mosquitoes. RNA was extracted from mosquitoes, blotted onto nitrocellulose, and hybridized with the probe. Using this technique, 100 ng of RNA was readily detected.
Biotinylated cDNA probes have also been prepared for dengue virus. One probe was capable of detecting picogram levels of dengue virus RNA. Some signal was detected in control cells. To investigate this phenomenom, the three dengue specific probes were labeled with ^{32}P. The use of isotopic probes revealed unequivocally that all probes contained dengue sequences.

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Summary

A molecular hybridization technique was developed to detect the S RNA of LaCrosse (LAC) virus in BHK-21 cells. A complementary DNA (cDNA) to the S RNA was cloned into the PvuII site of the plasmid pBR322. The recombinant plasmid was then labeled by enzymatic incorporation of biotinylated dUTP and used as a probe. Optimal hybridization and detection parameters were determined, resulting in the following protocol: cells grown to confluency on subbed slides and infected with LAC virus were fixed in paraformaldehyde, permeabilized with Triton X-100, and hybridized with the labeled, denatured probe overnight at 37C. After hybridization, cells were treated sequentially with the following reagents to detect hybridized probe: 1) goat anti-biotin antibody, 2) biotinylated rabbit anti-goat antibody, 3) avidin-biotinylated horseradish peroxidase, and 4) substrate (0.2mg/ml DAB - 0.06% H_2O_2).

Virus antigen was first detected by direct immunofluorescence in a few cells at 2 hours post infection; by 6 hours, most cells were expressing substantial antigen. The presence of specific hybrids was first detected in the perinuclear region of cells at 4 hours PI. By 6 hours, hybridization signal was detected in most cells. By 12 hours signal was detected in more distal portions of the cell, and by 24 hours intense hybridization signal was detected throughout the cytoplasm.

Three cultures of transformed <u>E. coli</u> containing recombinant plasmids bearing cDNA to dengue-2 virus genome segments were obtained from Dr. Radha Padmanabhan at the University of Kansas. The three clones pVV1 (1.4 kb dengue insert), pVV9 (1.65 kb insert), and pVV17 (1.95kb insert) were grown and amplified by standard procedures. Plasmids were extracted, quantified, and analyzed for the correct constructs by gel electrophoresis. The recombinant plasmids were biotin-labeled by nick translation in which dTTP was replaced by bio-11-dUTP.

The three biotinylated probes have been used in preliminary hybridization studies to determine sensitivity and specificity of each. Crude cell culture supernatant, cytoplasmic RNA preparations, and extracted viral RNA were blotted onto nitrocellulose and hybridized with each of the probes. Hybridized dengue cDNA was detected by immunobiochemical techniques. The largest probe, pVV17, has proven to be most sensitive, detecting picogram levels of extracted dengue-2 viral RNA with little reactivity with cellular RNA. The other probes, pVV1, and pVV9, appeared to react to a greater extent with cellular preparations. However, when the three inserts were isolated from the plasmids, nick translated using ^{32}P-dCTP, and hybridized to dengue virus RNAs blotted onto nitrocellulose, no cross reactions with cell preparations were noted. The pVV1 derived probe was the most sensitive and detected 32 pg of dengue-2 RNA, 160 pg of dengue-3, and 20 ng of dengue-4. Dengue-2 and dengue-3 RNAs were extracted from <u>Aedes albopictus</u> cells. The pVV9 and pVV17 probes were more specific for dengue-2 RNA, but some cross reaction with other dengue serotypes was noted.

Foreword

In conducting this research, the investigators have abided by the National Institutes of Health Guidlines for Research Involving Recombinant DNA molecules (May, 1986).

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BODY OF THE REPORT

I. Statement of the problem

Studies were proposed to develop, to evaluate, and to apply nucleic acid hybridization probe methodology to the surveillance and diagnosis of arboviruses in vectors or vector populations. Special attention was to be devoted to development of methods that can be applied in field circumstances. In particular, biotin-labeled hybridization probes (BLHPs) which can be detected by enzyme immunoassay techniques, were to be emphasized. Sensitivity and specificity of BLHP methods were to be compared and contrasted with conventional radioisotopic hybridization probe procedures as well as virus isolation and antigen detection techniques.

II. Background

Surveillance programs permit identification of geographical areas of high risk for arbovirus infections. Surveillance programs for arboviruses usually entail monitoring of either infections in humans and/or other vertebrates and monitoring of vector populations and infection rates (1). Estimates of vector population densities and infection rates are particularly useful for predicting the risk of human and animal disease. Since such information can be gathered before the incidence of human disease, health practitioners can successfully intervene to prevent infections.

The conventional method for arbovirus surveillance involves isolation of virus from mosquitoes or mosquito pools by amplification in a bioassay such as suckling mice, cell cultures, or embryonated eggs (1). Virus isolates must then be serologically identified. These conventional techniques are labor intensive, slow, and may require elaborate laboratory tissue culture and containment facilities. Frequently samples must be collected and sent to distant central laboratories for processing. Results typically are not available in time to permit field practitioners to institute effective control or therapeutic measures. Further, samples must be protected from environmental conditions which could destroy virus infectivity. Thus elaborate cold-chains or other protective measures are necessary to ensure virus viability for amplification.

Second generation diagnostic techniques have circumvented several of these problems. In these tests, diagnosis is effected by detection and simultaneous identification of virus specific antigens. Enzyme immunoassays (EIAs) are examples of second generation diagnostic procedures which preclude many difficulties associated with conventional virus isolation and subsequent identification techniques (1, 2). However, certain technical and methodological difficulties are inherent in EIA techniques. Many of these can be precluded by the use of a third generation diagnostic test, nucleic acid (NA) hybridization. Since direct detection of NA is the method of diagnosis, concerns with antigen-antibody equivalence, antigen clearance or degradation, latent infections or other infections without substantial antigen production, and specificity of immunoglobulins are all eliminated. Nonetheless, the sensitivity and specificity of EIA may be retained. A drawback to the widespread use of hybridization in diagnosis has been the need for radioisotopic labeling of probes. However, development of BLHPs by Ward and co-workers (3, 4) provides a technique that retains the exquisite sensitivity and specificity of nucleic acid hybridization without drawbacks associated with safety, shelf-life, and disposal of radioisotopes. After hybridization, the biotin reporter molecule incorporated into a probe can be detected immunologically by immunofluorescence (IF) or enzyme immunoassay (EIA). Biotinylated NA hybridization techniques have gained wide acceptance for detection of both integrated and extrachromosomal virus-specified NA sequences (3, 5-7). However these techniques had not been applied to the detection of arboviruses in vectors.

The rationale for this proposal was that NA hybridization techniques should be applicable to detection of arbovirus genomes or specified NA sequences in vectors. Further, NA hybridization should provide an extremely sensitive and specific mechanism for detection of infected arthropods. With the substitution of biotin-labeled NA probes for radioisotopic probes, the technique should be adaptable to field situations and should permit rapid diagnostic capability. Further, immobilization of virus NA on nitrocellulose should permit storage and transport of diagnostic samples, thereby precluding problems of preservation of virus antigens or infectious virus.

III. Methods and Results

A. LaCrosse (LAC) virus: Development of techniques for detection of virus nucleic acid (NA) species:

In the LAC virus system, progress has been made in both *in situ* hybridization and detection of LAC RNA in blots from cells and mosquito suspensions. In both types of specimens we have used as a probe the cDNA of the S RNA of LAC virus cloned into the PvuII site of the plasmid pBR322 (8). The probe was labeled with biotinylated-dUTP by nick translation.

1. *In situ* hybridization

The complete protocol used for *in situ* hybridization is published elsewhere (3). In brief, cells were propagated on subbed slides, infected with LaCrosse virus (MOI=10), fixed in paraformaldehyde, treated with Triton X-100, and then hybridized with the probe. After hybridization, slides were successively treated with goat anti-biotin Ab, biotinylated rabbit anti-goat Ab, ABC complex, and DAB. In infected cells, the converted substrate was seen as a brown precipitate in the cytoplasm of the cells.

The technique was used to determine the time and cellular localization of synthesis of the S RNA species of LAC virus in BHK-21 cells. Results were contrasted with immunofluorescent detection of virus antigen in the cells (Figures 1, 2, and 3). Hybridized probe was first detected 4 hours post infection; the signal was not intense and was restricted to distinct perinuclear areas. By 12 hours, strong signal was detected in perinuclear spaces and signal was detectable in more distal regions of the cytoplasm. By 24 hours, intense signal was detected throughout the cytoplasm. A few cells contained detectable virus antigen by 2 hours post infection. Most cells were expressing significant amounts of antigen by 4-6 hours.

2. Dot/blot hybridization

Dot/blot hybridization protocols were developed using LAC viral RNA extracted from supernatants of infected cell cultures. After PEG precipitation and centrifugation, viral pellets were resuspended in STE buffer containing SDS. After phenol:chloroform:isoamyl alcohol and subsequently chloroform:isoamyl alcohol extraction, the RNA was recovered by ethanol precipitation. The RNA was pelleted, dried in vacuo, and resuspended in 10xSSC. The concentration of RNA was determined by UV absorption. To prepare blots, dilutions of RNA were made in 15xSSC-7.4% formaldehyde, incubated at 65C for 15 minutes, then blotted onto nitrocellulose (10) using a slot blot apparatus. The nitrocellulose sheets were then baked in vacuo for 2 hours at 80C. Blots were pretreated to prevent nonspecific binding of probe, and then hybridized in 50% formamide, 5xSSC, 25 mM $NaPO_4$, 1xDenhardts solution, 250 ug/ml carrier DNA, 10% dextran sulfate, and 0.1ug/ml labeled probe for 16 hours at 42C. After hybridization, sheets were washed, blocked, and the detection system applied. Detection steps included steptavidin , biotinylated poly-alkaline phosphatase, and NBT-BCIP substrate. These were added for 10 minutes, 10 minutes, and 3-4 hours, respectively, with intermittent washes. Development of color was stopped by washing the blots in TRIS-EDTA.

Mosquito suspensions were similarly treated with the exception of the exclusion of PEG precipitation and centrifugation. Mosquito processing began with the phenol:chloroform extraction step.

When LAC virus NA is blotted onto nitrocellulose, 100 ng of RNA can be detected readily (Figure 4). However, this technique undoubtedly greatly underestimates the sensitivity of the probe. For example, the efficiency of LAC RNA binding to the nitrocellulose is not known. In this procedure, probe only detects S RNA species; L and M species are not detected. In addition, the blot represents only a crude cell culture supernatant extract; thus the estimated amount of RNA probably includes cellular RNA. Nonetheless the results are encouraging.

As expected, the LAC S RNA cDNA probe does not differentiate between California group viruses (Data not shown). S RNA species from snowshoe hare, trivittatus, and tahyna viruses cross-hybridize. The lack of specificity with the S RNA species is not unexpected, because it is the most conserved portion of the genome.

The probe was also capable of detecting S RNA species in infected mosquitoes (Figure 5). In these preliminary studies, two pools each of 10 infected or noninfected mosquitoes were processed as described. After extraction, RNA from infected and noninfected mosquito suspensions was blotted onto nitrocellulose and processed as noted previously. As can be seen in Figure 5, substantial signal resulted from the infected pools. Some signal was detected in the negative pools, but the intensity of the signal was dramatically higher in the positive pools.

B. Dengue virus: development of techniques to detect NA species:

For detection of dengue virus by hybridization, most effort has been directed to detection of virus nucleic acids in suspension. Both biotinylated and radiolableled cDNA and RNA probes have been used to detect dengue virus in blot hybridization protocols.

1. Dengue blot hybridization: biotinylated probes

Blot hybridization techniques have also been developed for the detection of dengue virus RNA species in cell extracts. Three plasmids containing cDNA sequences of the dengue-2 virus genome cloned into the Pst I site of pUC13 were obtained from Dr. R. Padmanabhan of the University of Kansas. The three recombinant plasmids, pVV1 (1.4 kb insert), pVV9 (1.65 kb insert), and pVV17 (1.95 kb insert) were biotin labeled by nick translation with biotin-11-dUTP. The probes were compared for sensitivity and specificity in hybridization studies using crude cell culture supernatants, cytoplasmic RNA preparations, and extracted viral RNA. The probes were hybridized to the respective samples blotted onto nitrocellulose (9). The largest probe, pVV17, has proved thus far to be the most sensitive, detecting picogram levels of extracted dengue-2 viral RNA with little cross reactivity. For unknown reasons, the other two probes exhibited greater cross reactivity with cellular extracts.

2. Dengue blot hybridization - radiolableled cDNA probes

To investigate further the specificity of the probes, the three cDNA inserts were isolated from the plasmids and nick translated using ^{32}P-dCTP. These probes were hybridized to RNAs extracted from purified virus and infected C6/36 mosquito cell lysates. RNAs extracted from uninfected cell lysates served as one negative control and blotting of solutions containing no RNA served as another. RNAs were blotted onto nitrocellulose using a dot blot manifold apparatus in high salt conditions (10xSSC). Following denaturation in the presence of formaldehyde (7.4%) at 65C for 15 minutes, blots were baked at 80C for 2 hours. Hybridization was at 42C for 48 hours. For autoradiography, blots were exposed to x-ray film overnight at -70C. Longer exposures may well increase the sensitivities noted below.

The hybridization results corroborate that all 3 clones contained dengue specific inserts that strongly hybridized to dengue-2 RNA and to the RNA of other dengue serotypes to varying degrees (Figure 6). The pVV1 derived probe detected 32 pg of dengue-2 RNA, 160 pg of dengue-3 viral RNA, and 20 ng of dengue-4 RNA. The pVV1 probe also detected dengue RNA sequences extracted from dengue 2 and 3 infected C6/36 cells. The pVV9 probe was dengue-2 specific and detected 4 ng of RNA. The pVV17 probe was primarily dengue-2 specific, detecting 800 pg of viral RNA. Some hybridization was noted with RNAs extracted from dengue 1 and 3 viruses. The pUC-13-1 probe (non-recombinant plasmid control) only hybridized to pUC-8 and not to any RNAs, indicating that the pUC-13-1 plasmid vector is not a source of nonspecificity. None of the probes hybridized to the negative controls. Thus, non-specific binding of plasmid probes can not be attributed to either the vector DNA or the cDNA inserts and may instead be due to the system used for detection of hybrids. Also, the cDNA sequences vary in both specificity and sensitivity for detection of dengue-2 and the other dengue virus serotypes.

VI. Discussion

Significant progress has been made in this first granting period, especially in the areas of development and characterization of constructs and protocol development. Techniques for blotting and detecting LAC and dengue RNA species on nitrocellulose using biotinylated probes have been developed, as well as techniques to detect LAC RNA *in situ.* Results thus far are most encouraging. However, two major problems (or potential problems) have been encountered which impede the use of hybridization in virus diagnosis and surveillance, especially in clinical or field situations. These are problems associated with sensitivity for detection of nucleic acid blotted onto nitrocellulose and the need for laborious, time consuming extraction procedures necessary to purify the RNA for processing in the current protocols.

For detection of dengue virus RNA immobilized on nitrocellulose, the sensitivity level is 1.5×10^6 genome equivalents. For LAC virus RNA even a greater concentration is required. Yet for LAC *in situ* hybridization, strong signal is detectable in cells when the titer is less than 3.0 log $TCID_{50}$ per ml. Thus, there seems to be an inherent problem involved in retaining RNA which has been blotted directly to nitrocellulose.

In our hands, phenol-chloroform extraction procedures have proven to be essential to obtain consistent, sensitive results. Such laborious and extensive procedures would be ill-suited to field or clinical situations.

Because of these difficulties or potential problems, we have embarked upon the development of sandwich hybridization techniques to detect arbovirus NA in mosquito suspensions. These techniques appear to be more sensitive than conventional blot hybridization, presumably because RNA need not be immobilized on nitrocellulose. We have not determined the efficacy of the technique for detection of NA in mosquito suspensions. This will be emphasized in the next granting period, along with studies to compare and contrast the diagnostic efficacy with conventional techniques.

V. Cost Analysis (curve) of budgetary expenditures:

Projected and actual budget expenditures are noted for this past year of the contract and projected expenditures are noted for the period Oct 1, 1986 to Sept 30, 1987 (Figure 7). No significant budgetary deficits or balances are anticipated.

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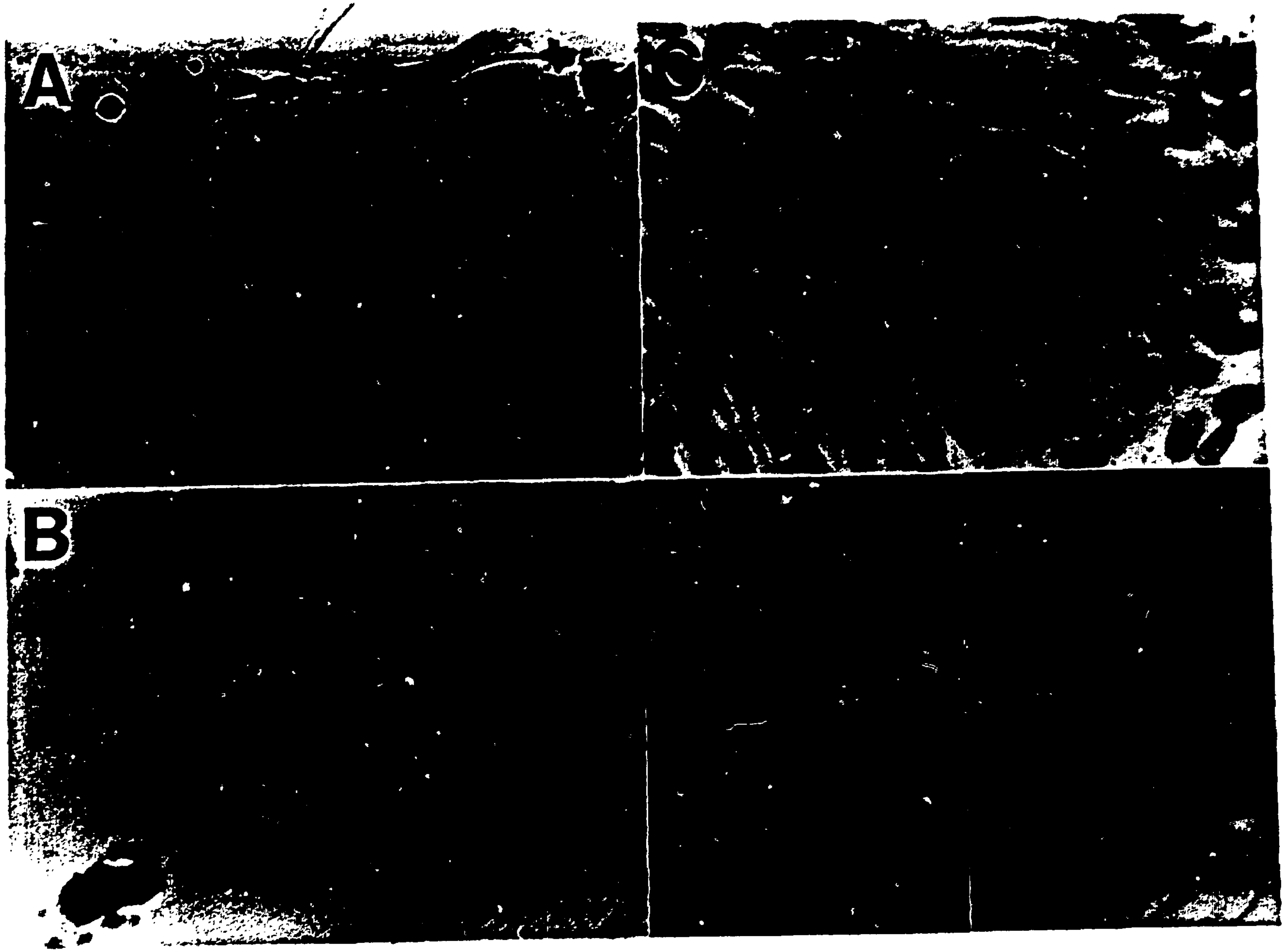


DETECTION OF LACROSSE VIRUS AT 6 HOURS POST INFECTION:

A. VIRUS S RNA BY *IN SITU* HYBRIDIZATION (400X)

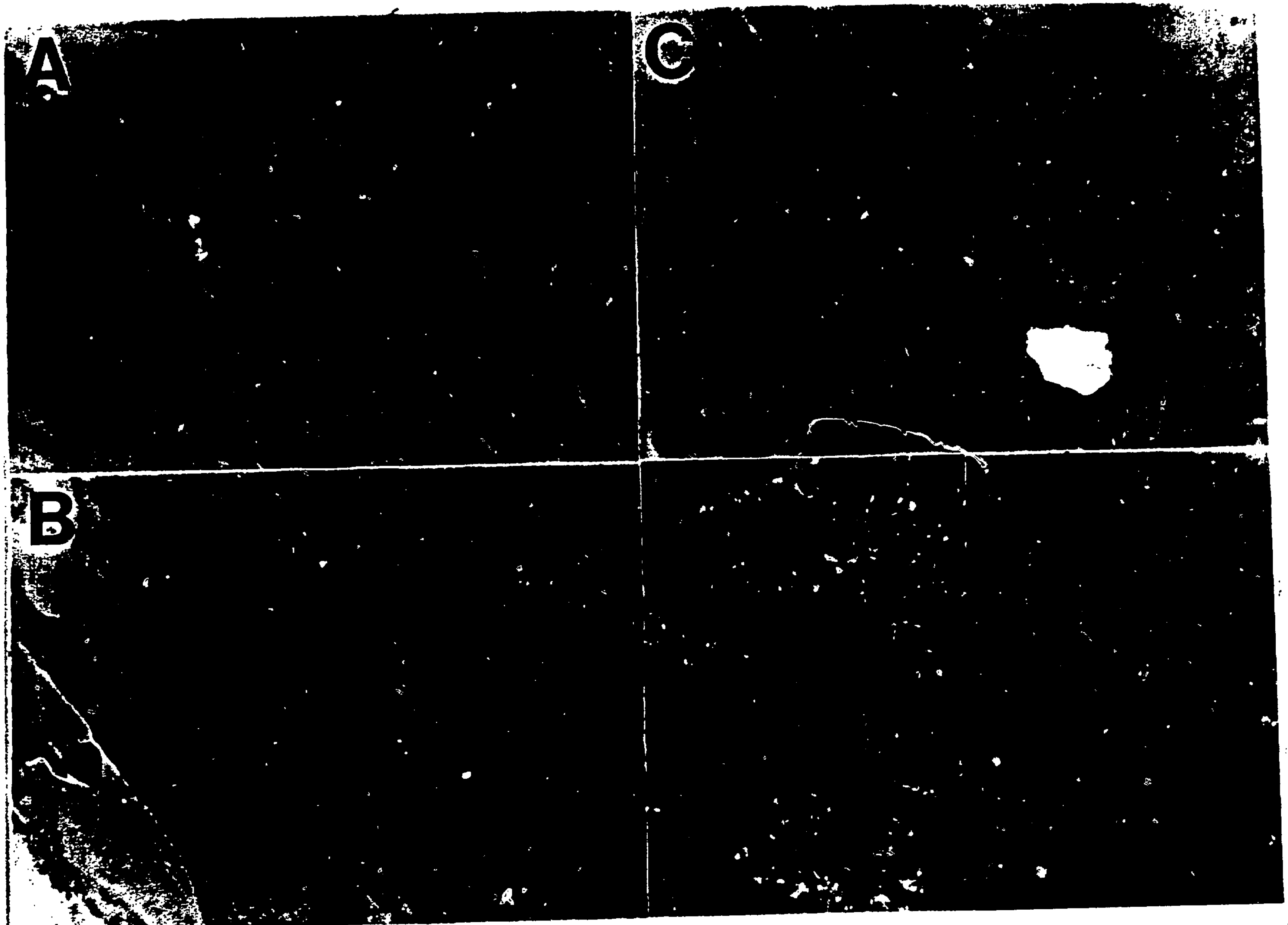
B. VIRUS S RNA BY *IN SITU* HYBRIDIZATION (1000X, NOMARSKI OPTICS)

C. UNINFECTED CELLS - HYBRIDIZATION CONTROL (400X, NOMARSKI OPTICS)

D. VIRUS ANTIGEN BY IMMUNOFLUORESCENCE (400X)

E. UNINFECTED CELLS - IMMUNOFLUORESCENCE CONTROL (1000X)

FIG. 1



DETECTION OF LACROSSE VIRUS AT 12 HOURS POST INFECTION:

A. VIRUS S RNA BY IN SITU HYBRIDIZATION (1000X)

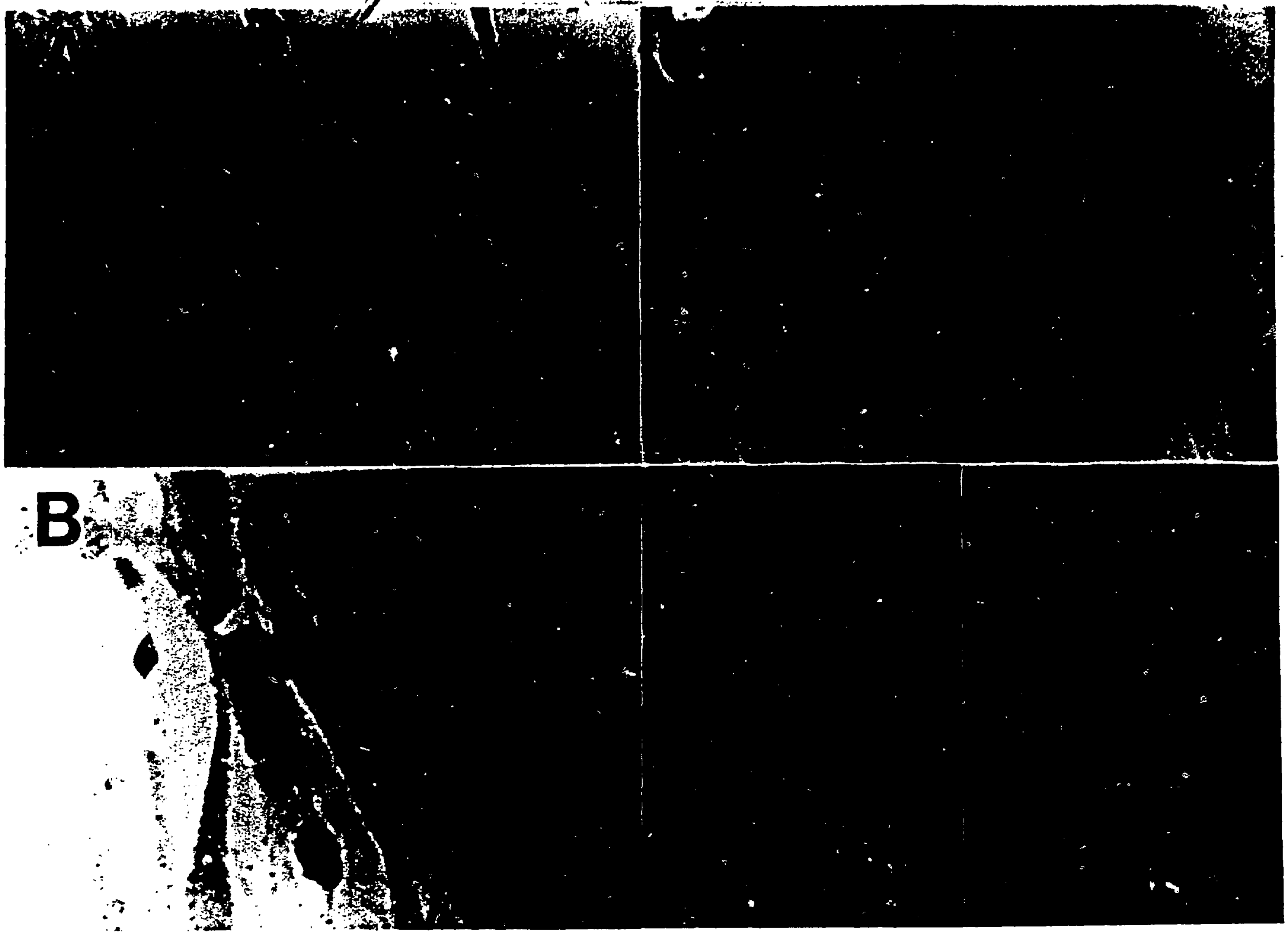
B. VIRUS S RNA BY IN SITU HYBRIDIZATION (1000X, NOMARSKI OPTICS)

C. UNINFECTED CELLS - HYBRIDIZATION CONTROL (1000X, NOMARSKI OPTICS)

D. VIRUS ANTIGEN BY IMMUNOFLUORESCENCE (400X)

E. UNINFECTED CELLS - IMMUNOFLUORESCENCE CONTROL (1000X)

FIG. 2



DETECTION OF LACROSSE VIRUS AT 24 HOURS POST INFECTION:

A. VIRUS S RNA BY _IN SITU_ HYBRIDIZATION (1000X)

B. VIRUS S RNA BY _IN SITU_ HYBRIDIZATION (1000X, NOMARSKI OPTICS)

C. UNINFECTED CELLS - HYBRIDIZATION CONTROL (1000X, NOMARSKI OPTICS)

D. VIRUS ANTIGEN BY IMMUNOFLUORESCENCE (1000X)

E. UNINFECTED CELLS - IMMUNOFLUORESCENCE CONTROL (1000X)

FIG. 3

Figure 4

Detection of LaCrosse virus nucleic acid in cell culture suspensions using a biotinylated cDNA probe

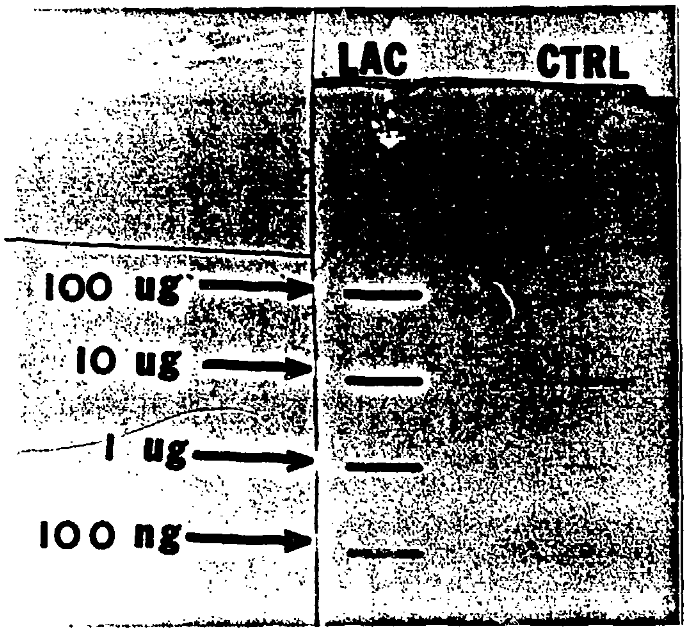


Figure 5

Detection of LaCrosse virus nucleic acid in mosquito suspensions using a biotinylated cDNA probe

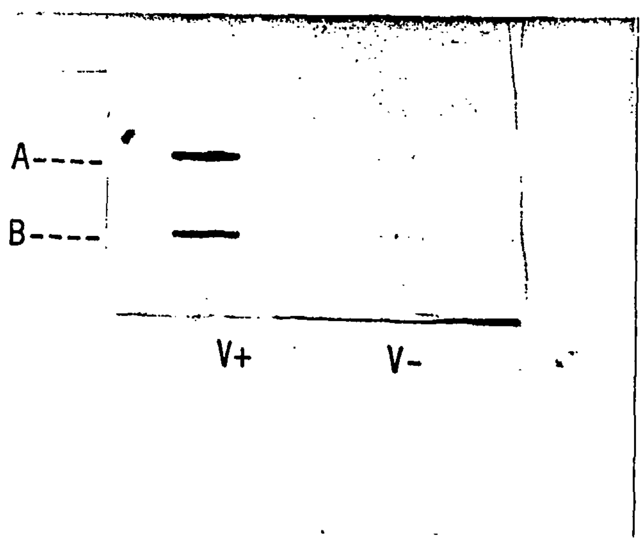


FIGURE 6 COST CURVE